FACULTAD DE MEDICINA

DEPARTAMENTO DE MEDICINA

"Diabetes mellitus: Impact on the pathophysiology, diagnose and treatment of the hepatic encephalopathy"

TESIS DOCTORAL

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Unidad de Gestión Clínica de Enfermedades Digestivas

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CERTIFICA:

Que la Tesis Doctoral que lleva por título: ***"Diabetes mellitus: Impact on the pathophysiology, diagnose and treatment of the hepatic encephalopathy"***, presentada por D. Javier Ampuero Herrojo para optar al grado de Doctor, ha sido realizada en el Departamento de Medicina de la Facultad de Medicina de la Universidad de Sevilla.

Revisado el texto, doy mi conformidad para su presentación y defensa para optar al grado de Doctor por la Universidad de Sevilla.

Fdo. Dr. Manuel Romero Gómez

DIRECTOR DE LA TESIS

26 de Mayo de 2014

D. JOSÉ VILLAR ORTIZ, CATEDRÁTICO DE

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Tesis por compendio de publicaciones

En el actual contexto universitario, la calidad de la formación así como de los resultados de la misma tiene un papel relevante. Teniendo en cuenta que una de las competencias básicas que debe adquirir el doctorando es la capacidad de comunicación con la comunidad académica y científica, las publicaciones en revistas especializadas garantizan la adquisición de esta competencia, además de otras relacionadas con ella.

Por ello, y con el consentimiento de mi director, he creído conveniente que la Tesis Doctoral que aquí se presenta tenga este formato por las siguientes razones: a) porque permite comunicar a la comunidad científica los resultados obtenidos de una forma rápida, no teniendo que esperar varios años hasta la finalización del proyecto de tesis, lo cual puede provocar que los hallazgos pierdan originalidad e interés; b) porque uno de los principales criterios por los que se mide la calidad de un trabajo científico es a través del nivel de impacto de las revistas en las que éste es publicado. El conjunto de trabajos presentados en esta Tesis, y que he realizado con la ayuda de mi director y otros compañeros, se ha publicado en revistas internacionales con impacto, tras estrictas revisiones llevadas a cabo por expertos en el área de la Hepatología, lo cual creemos que es motivo de satisfacción, puesto que se trata de un importante indicador de la calidad del trabajo realizado.

Los miembros del tribunal comprobarán que los trabajos publicados están firmados por varios autores. Este hecho se debe a que dichos trabajos han sido realizados en colaboración con otros compañeros de distintos departamentos, sin los cuales la tarea habría sido difícilmente abordable.

Informe de las publicaciones

Los artículos de los que consta esta Tesis por compendio de publicaciones son:

-**Ampuero J**, Ranchal I, Núñez D, Díaz-Herrero MM, Maraver M, Del Campo JA, Rojas A, Camacho I, Figueruela B, Bautista JD, Romero-Gómez M.

- Metformin inhibits glutaminase activity and protects against hepatic encephalopathy.

- PLoS One 2012; 7(11): e49279. doi: 10.1371/journal.pone.

- Factor de impacto 4.092. ISSN: 1932-6203.

-**Ampuero J**, Ranchal I, Del Mar Díaz-Herrero M, Del Campo J, Bautista J, Romero-Gómez M**.**

- Role of diabetes mellitus on hepatic encephalopathy.

- Metab Brain Dis 2013; 28: 277-9.

- Factor de impacto 2.333. ISSN 1573-7365.

- Romero-Gómez M, **Ampuero J**.

- Deciphering the spectrum of low-grade hepatic encephalopathy in clinical practice.

- Gastroenterology 2014; 146: 887-90.

- Factor de impacto 12.821. ISSN: 0016-5085.

Así mismo, se considera oportuno incluir en los apéndices de la Tesis los siguientes artículos y capítulos que han constituido parte de la base formativa de la misma:

-**Ampuero J**, Romero-Gómez M.

- Manejo actual de la encefalopatía hepática.

- RAPD Online Vol 35. Nº5 Suplemento. Octubre 2012. 378-385.

- ISSN 1988-317X.

-**Ampuero J**, Romero-Gómez M.

-Oral Glutamine Challenge. Glutamine in Health and Disease. King's College London: Springer; 2014 (*in press).*

Contents

Abbreviations

BT: Bacterial translocation.

CFF: Critical flicker frequency.

COX: Cyclooxigenase.

CT: Computerized tomography.

DM: Diabetes mellitus.

EEG: Electroencephalography.

HCV: Hepatitis C virus.

HE: Hepatic encephalopathy.

HR: Hazard ratio.

ICT: Inhibitory control test.

IGF: Insulin like growth factor.

IL: Interleukin.

IRS: Insulin receptor substrate.

MHE: Minimal hepatic encephalopathy.

MRI: Magnetic resonance imaging.

NAFLD: non-alcoholic fatty liver disease.

NASH: non-alcoholic steatohepatitis.

OR: Odds ratio.

PHES: Psychometric hepatic encephalopathy score.

PPAR: Peroxisome proliferator-activated receptor.

RBANS: Repeatable Battery for the Assessment of Neurological Status.

RR: Relative risk.

SIBO: Small intestinal bacterial overgrowth.

TIPS: Transjugular intrahepatic porto-systemic shunt.

TNF: Tumor necrosis factor.

TZD: Thiazolidinediones.

Introduction

Liver cirrhosis

ETIOLOGY AND EPIDEMIOLOGY

Liver fibrosis is the excessive accumulation of extracellular matrix in response to acute or chronic liver injury. Liver cirrhosis is the final stage of liver fibrosis, characterized by the histological development of regeneration nodules surrounded by fibrous tissue, and leading to a loss of normal liver function and appearing metabolic and hemodynamic disturbances[1]. The main causes of cirrhosis are alcoholic liver disease, hepatitis C (HCV), hepatitis B, non-alcoholic steatohepatitis (NASH), autoimmune hepatitis and primary biliary cirrhosis[2]. Interestingly, the etiology of cirrhosis differs across the world. Viral hepatitis is the leading cause of cirrhosis in developing countries and alcoholic liver disease, HCV and NASH are the most significant causes of cirrhosis in the developed countries[3].

In many developed countries, the death rates from liver cirrhosis have been declining in the recent years; especially those derived from alcohol-related cirrhosis. By contrast, an increase has been observed in a few Eastern European countries and England[4]. In United States, there has been an increase by HCV compared to alcoholic liver disease. Data about patients' characteristics at diagnosis show that the mean age is around 60 years, being more frequently in male sex. The highest mortality from liver cirrhosis is in the age group 60-70 years[5].

DIAGNOSTIC METHODS

The diagnosis of cirrhosis is based on a combination of clinical, ultrasound, biochemical and histological findings **(Table 1)**. Liver biopsy is still considered the

"gold standard" for the diagnosis of cirrhosis, but is being less employed over time. A liver biopsy shows some procedural risks, including bleeding, considerable discomfort for the patient and even a risk of death[6]. Consequently, several other non-invasive methods for diagnosis are being more commonly employed and evaluated. These include transient elastography, magnetic resonance elastography and combinations of ultrasound, computerized tomography (CT) and magnetic resonance imaging (MRI). In addition, combinations of tests and a number of non-invasive models for predicting liver fibrosis have been developed[7].

Imaging modalities	Ultrasonography
	CT/MRI
	Transient elastography
	Acoustic radiation force impulse imaging
	MR elastography
Indirect serum non-invasive fibrosis tests	APRI
	FIB-4
	Forns index
Proprietary serum non-invasive fibrosis tests	Fibrotest
	ELF
	Hepascore
	Fibrometer

Table 1. Diagnostic methods for liver cirrhosis.

CLINICAL RELEVANCE

Cirrhosis affects hundreds of millions of patients worldwide. It is the final stage of liver fibrosis as a result of the disorganization of the connective tissue and the regeneration of hepatic parenchymal cells[8]. In the evolution of many chronic liver

diseases, cirrhosis is a stage that has been considered irreversible although the evidence that fibrosis and even cirrhosis can be reversible is growing[9]. The chronic liver disease promotes an underlying immunological response which causes persistent inflammation and tissue repairing and, ultimately, leads to fibrosis and cirrhosis. This latter can be stabilized controlling the primary disease (e.g. antiviral therapy, immunosuppressive therapy, exercise). However, the presence of liver cirrhosis can involve an increased risk of developing portal hypertension and intrahepatic shunting of blood, together with impaired protein synthesis and hormone metabolism[10]. Therefore, clinical manifestations of cirrhosis range from asymptomatic to symptomatic depending on the nature and the severity of the underlying liver disease. Over time, liver cirrhosis is associated with severe complications which lead to decompensate cirrhosis from compensated cirrhosis[11]. These are variceal bleeding, ascites, hepatic encephalopathy, hepatorenal syndrome, bacterial infections such as spontaneous bacterial peritonitis and hepatocellular carcinoma. Patients suffering such complications show 50% of 5-year mortality.

Hepatic encephalopathy

INTRODUCTION

Hepatic encephalopathy (HE) is one of the major complications of liver cirrhosis, affecting up to one third of cirrhotic patients. HE can be defined as a complex neuropsychiatric disturbance that occurs in patients with liver dysfunction. It ranges from minimal behavioral abnormalities to deep coma. The clinical prognosis is poor after HE has developed, with 1-year survival estimated at 42% and 3-year survival at 23%[12]. HE is classified in three types: type A, associated with acute liver failure; type B, related to porto-systemic shunting without intrinsic liver disease; and type C, linked to cirrhosis[13] **(Table 2)**. Of all these types, HE associated with liver cirrhosis is the most prevalent. It is often the result of hepatic parenchymal damage or increased porto-systemic shunting. However, one or more precipitating factor can be identified in about 60%-70% of cases[14]. HE in cirrhosis can be treated satisfactory, either by non-absorbable disaccharides or antibiotics, or by eliminating the precipitating factors. Ultimately, liver transplantation is available as the last option in those cirrhotic patients with liver insufficiency and recurrent episodes of HE[15]. However, we can identify two major groups of cirrhotic patients with an increased risk of chronic recurrent episodes of HE, and which cannot be satisfactory treated with the current treatment. Firstly, patients showing contraindications for liver transplantation (i.e. old cirrhotic patients, patients treated with a transjugular intrahepatic porto-systemic shunt (TIPS)). Secondly, 30%-50% of cirrhotic patients show minimal hepatic encephalopathy (MHE)[16]. This entity represents the first stage in HE spectrum (**Figure 1**). MHE is defined as the presence of cognitive abnormalities in patients with liver disease, which are not detected with common examinations; it is diagnosed using sensitive neuropsychological and

neurophysiological tests[17]. It is characterized, additionally to cognitive impairment, by decreased attention, poor concentration, impaired memory, sleep disturbances and psychomotor slowing. Furthermore, MHE predicts the appearance of HE and survival in cirrhotic patients[18], as well as increases the risk of having a car accident[19] and falls[20].

Type	Definition
A	Acute and hyperacute liver failure
B	Portosystemic bypass without intrinsic hepatocellular disease
C	Cirrhosis and portal hypertension with portosystemic shunts

Table 2. Classification of hepatic encephalopathy.

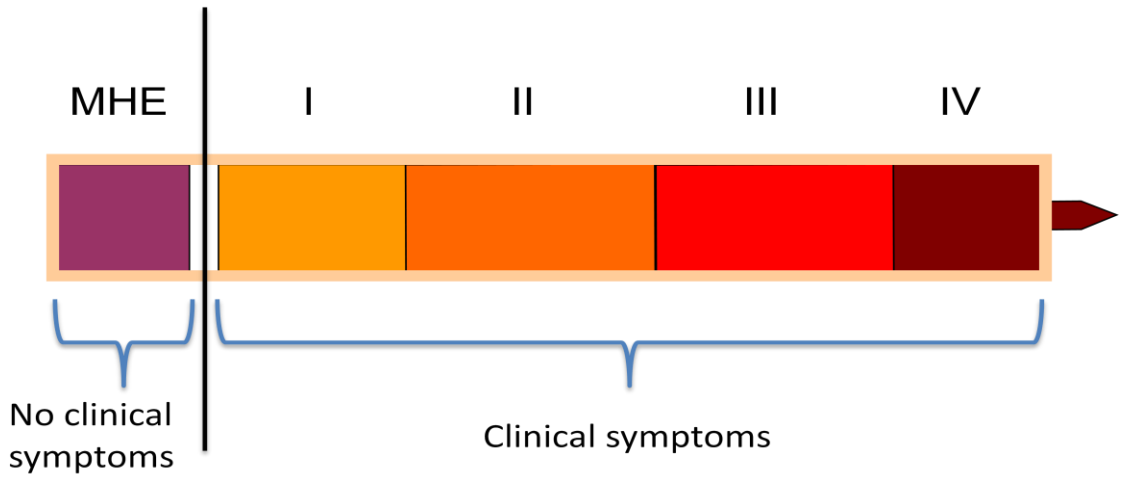

Figure 1. Spectrum of hepatic encephalopathy.

PATHOGENESIS

The pathophysiology of HE is multifactorial and complex, and it remains poorly understood. Traditionally, hyperammonemia has been considered the cornerstone of

HE. However, over the past decade, coexisting factors have emerged, such as the systemic inflammatory response or the presence of genetic factors, which seem to have a synergistic role in pathophysiology of HE (**Figure 2**).

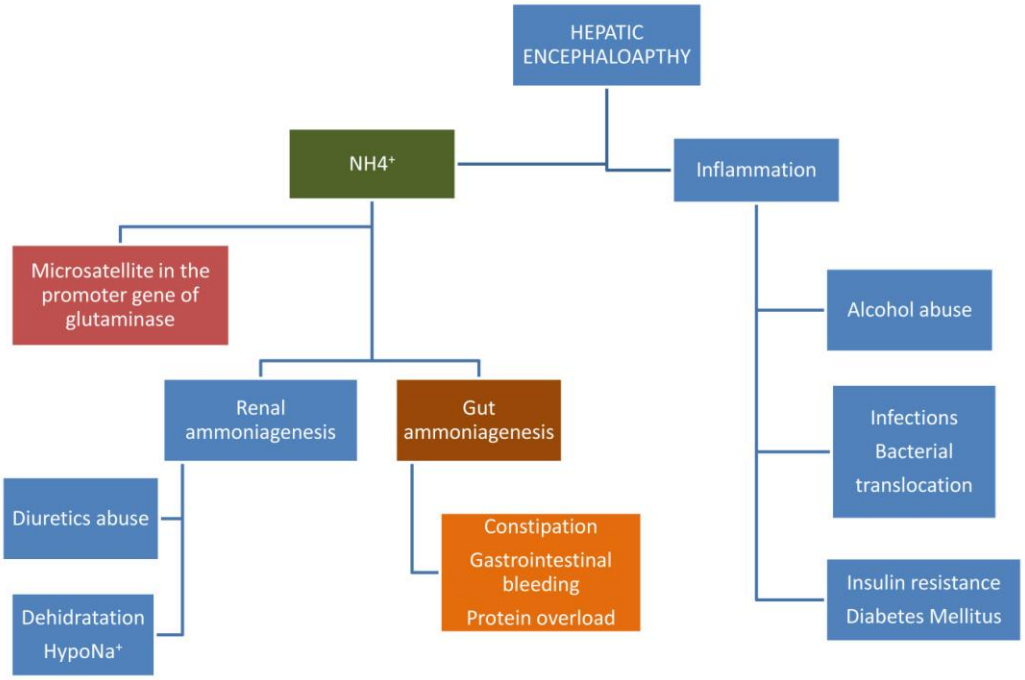

Figure 4. Potential hyperammoniagenic conditions.

Role of the ammonia

Ammonia is a normal physiologic product of intermediary metabolism, compound of nitrogen and hydrogen (**Figure 3**). The human body has several sources of ammonia. It is produced, mainly, after glutamine deamidation by glutaminase in small intestine (particularly in duodenum), as well as the urea hydrolyzed by gut bacteria[21]. Three glutaminase isoforms have been described: kidney type glutaminase, which is expressed in many mammalian tissues; liver type glutaminase, which was originally identified in hepatocytes; and type C present in peripheral mononuclear cells[22]. The

intestinal activity of glutaminase has been found to be higher in cirrhotic patients than in healthy controls[23].

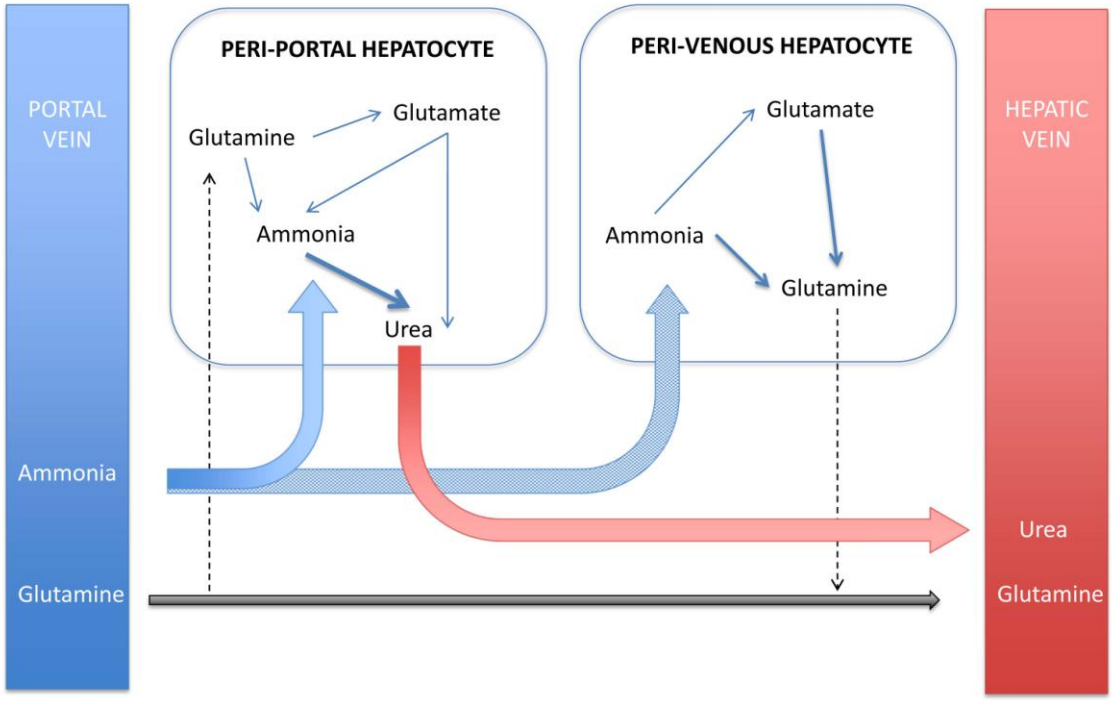

Figure 5. Ammonia detoxification: glutamine cycling and ureagenesis.

Ammonia is toxic at elevated concentrations and must be removed from the body (**Figure 4**). In fact, ammoniagenic conditions (ingestion of a protein load, constipation or a gastrointestinal bleeding) often result in HE, because of an elevation of ammonia concentration in the brain. Normally, the hepatic metabolism of ammonia takes place in two parts: a) the periportal hepatocyte, through the urea cycle (the most important step); b) near the central vein, hepatocytes transform the rest of ammonia into glutamine[24]. In cirrhosis or in presence of portosystemic shunts, the ammonia detoxification takes place in muscle, through glutamine synthesis (by glutamine synthetase). A hypoproteic diet decreases both muscle mass and ammonia detoxification. Thus, muscle and, consequently nutritional status, play an essential role

in cirrhotic patients[25] **(Table 3)**. On the other hand, the kidney is an organ capable of synthesizing and degrading ammonia. In liver disease, 70% of the synthesized ammonia is excreted in the urine, while decreases up to 30% of the ammonia under normal conditions[26]. Therefore, a renal failure may aggravate the cognitive status during HE.

	Glutaminase	Glutamina-synthetase
Low protein	--	--
High protein	++	=
Starvation	++	-
Diabetes mellitus	++++	+

Table 3. Regulation of hepatic glutaminasa and glutamine synthetase.

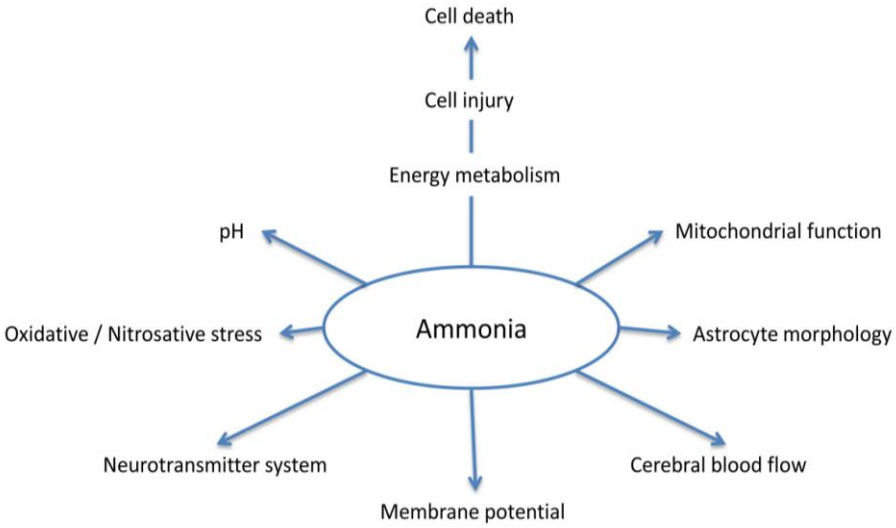

Figure 4. Pleomorphic effects of ammonia.

On the other hand, astrocytes are the only cells which metabolize ammonia in the brain. As muscles, they have glutamine synthetase and are able to convert glutamate and ammonia in glutamine, moving water into the astrocyte (glutamine is an

osmocyte)[27]. As a result, the astrocytes swell, and this process leads to cerebral edema and intracranial hypertension[28] **(Figure 5)**. However, this situation is more usual in acute liver failure than in chronic liver failure[29]. After a long exposition to ammonia, astrocytes change their morphology into Alzheimer's type II astrocytes[30], whose number correlates with the encephalopathy intensity[31]. This situation (brain edema) is compensated by the releasing of some osmolytes, such as myoinositol and taurine (homeostatic mechanism)[32]. On the other hand, hyperammonemia also produces reactive oxygen species in the central nervous system. Reactive oxygen species induces astrocyte swelling and mobilization of Zn, which mediates mRNA expression of metallothionein and the peripheral benzodiazepine receptor[33]. Resting of membrane potential or suppression of inhibitory postsynaptic potential formation are other effects of ammonia on the neural cells[34].

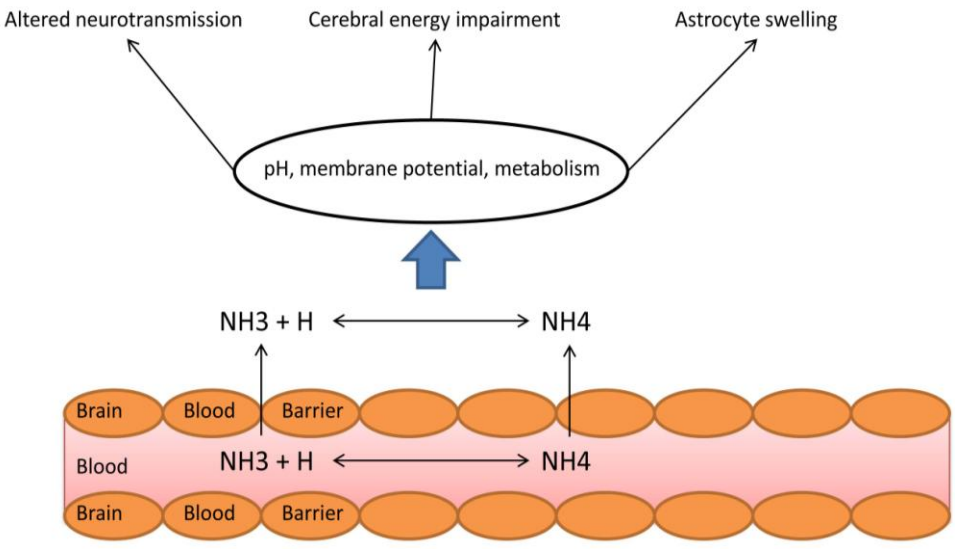

Figure 5. Mechanisms and effects of ammonia uptake into the brain.

Systemic inflammatory response

Systemic inflammation is common in liver failure and its acquisition is a predictor of hepatic encephalopathy severity[35]. An efficient recognition of systemic inflammation may change the natural history of HE and contribute to reduce circulating endotoxemia and immune cell dysfunction by developing novel therapeutic strategies.

Neuroinflammation

Pathophysiological mechanisms of HE are closely related to changes on the blood-brain barrier (made of endothelial cells and astrocytes), a highly specialized separation of brain microvasculature[36]. It is essential for the proper function of the central nervous system; among others, protects the brain from many common bacterial infections or toxins and from fluctuations in the blood plasma components. During infection, microglia cells (the resident macrophages of the brain) are activated and astrocytes release pro-inflammatory cytokines (tumor necrosis factor (TNF) α, interleukin (IL)-6), which enhance neuropsychological impairments induced by hyperammonemia[37]. Shawcross et al. reported the impact of systemic inflammatory response on ammonia-induced brain dysfunction in cirrhotic patients[38]. Several studies have documented that TNFα levels are higher in cirrhotic patients than in healthy subjects, with a positive correlation between them and HE severity[39]. Normally, the blood-brain barrier remains intact, preventing the entry of cytokines into the brain. However, pro-inflammatory cytokines (e.g. TNFα and IL-1) may produce brain swelling by inducing an increase in the permeability of the blood brain barrier[40]. In fact, treatment based on a TNF receptor antagonist (etanercept) reduces the severity of HE and prevents brain edema[41]. Treatment with infliximab, another monoclonal antibody against TNF, has also been shown to delay the progression of HE in rats[42]. Similarly, serum levels of IL-6 are correlated with the presence of MHE and its severity[43], as well as the cognitive function[44]. Precipitating factors for HE, such as

sepsis, hyponatraemia, gastrointestinal haemorrhage and renal failure, are known to increase production of TNFα[45]. Moreover, the therapeutic effect of hypothermia and non-steroidal anti-inflammatory drugs has been found to protect from central pro-inflammatory processes in mild HE, reducing the activation of microglial cells, which supports the concept of neuroinflammation[46]. Indeed, cyclooxygenase inhibitors, which were investigated in fulminant hepatic failure in a rat model, have shown that cyclooxygenase-1 rather than cyclooxygenase-2, plays a major role in HE, due to decreases the mortality and improves motor activities[47].

Bacterial translocation

Bacterial infections are well-known triggers for HE in patients with cirrhosis[48]. They are frequent complications in patients with cirrhosis (80% are caused by Gram-negative bacilli). Bacteria go through the gastrointestinal tract to extraintestinal sites, such as the mesenteric lymph node complex, liver, spleen, kidney and bloodstream, developing spontaneous bacterial peritonitis or bacteraemia[49] (**Figure 6**). Bacterial translocation (BT) may be caused by viable bacteria, as well as by fragments (endotoxins) or bacterial DNA. The following factors have been implicated in the appearance of BT: impaired immunity, small intestinal bacterial overgrowth (SIBO) and increased intestinal permeability[50]. Therefore, inflammatory response (derived from BT) may have a role in the pathogenic mechanisms involved in HE, due to circulating endotoxins produce TNFα and others cytotoxic molecules by activating macrophages[51]. Moreover, Jung et al. observed a very high frequency of SIBO in cirrhotic patients, showing a high correlation with BT and suggesting that SIBO could be a major risk factor of BT[52]. Hung et al. concluded that infections increase the mortality by HE in cirrhotic patients, especially pneumonia and sepsis without specific focus, after analyzing 4,150 adult cirrhotic patients hospitalized[53]. Recently, these

findings have been confirmed by Merli et al., who have documented a cognitive impairment (overt or subclinical) in 42% of cirrhotic patients without infection, in 79% with infection without SIRS and in 90% with sepsis[54]. Therefore, we should be cautious with bacterial infections in cirrhotic patients.

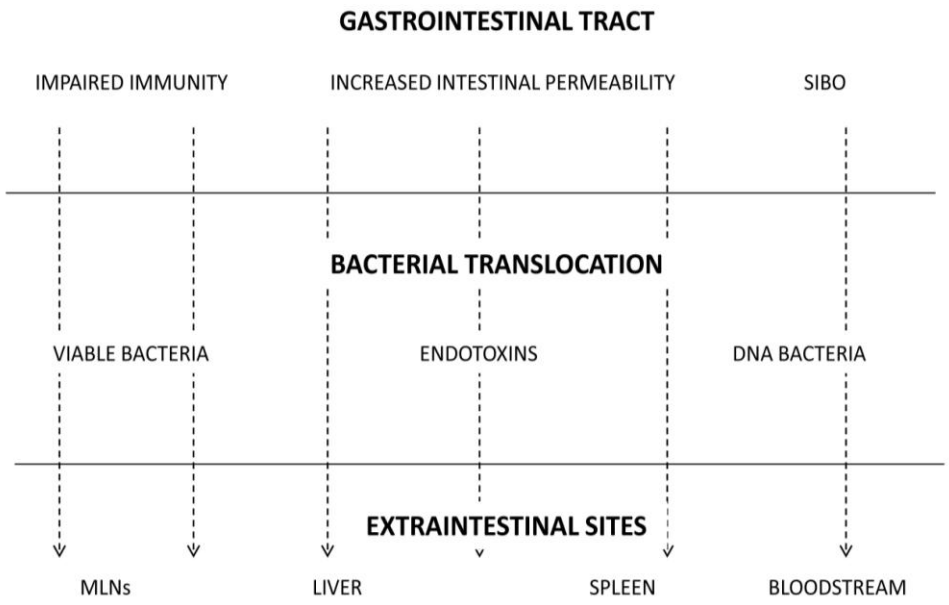

Figure 6. Mechanisms of bacterial translocation from gastrointestinal tract.

Genetic factors

Over time, genetic factors have been growing in importance in several fields. Data support the existence of genetic factors influencing the development of overt HE, due to cirrhotic patients showing the same degree of liver dysfunction (and suffering from the same trigger) may, or may not, develop overt HE[55]. Recently, Gorg et al. have carried out the first analysis of expression profiles of the whole transcriptome in *post mortem* cerebral cortex samples of cirrhotic patients with or without HE in comparison with patients without liver or neurological disease. The study showed a total of 638 or 396 altered genes in patients with cirrhosis with or without HE, respectively, compared

with controls without cirrhosis. They observed increased mRNA expression levels of HO-1, as well as other mRNA species involved in anti-oxidative defense (peroxiredoxin-4, selenoprotein-V, and PPARα) but also in glucose metabolism, in patients with cirrhosis and HE but not in those without HE. Thus, these findings indicate that gene expression profiles can discriminate cirrhotic patients with HE from those without HE and support the role of impaired insulin signaling and oxidative stress in the pathogenesis of this entity[56]. The genotype B1B1 of the TNFβ gene has been reported underexpressed in patients with HE after acetaminophen intoxication[57], but has not been confirmed in further studies. A polymorphism in the promoter region of the CD14 gene (-159C/T) was associated with HE, due to a strong relationship with advanced liver disease and systemic inflammation[58]. Nevertheless, all these association require confirmation in further studies.

The human glutaminase gene (OMIM: 138280) is located on chromosome 2 (2q32-q34). The full-length gene includes 84,675 basepairs and the glutaminase mRNA has 4784 basepairs (GenBank NM_014905)[59] **(Figure 7)**. In a prospective study (109 patients with cirrhosis in the estimation cohort, 177 patients in the validation cohort, and 107 healthy controls), Romero-Gomez et al. identified a microsatellite in the promoter region of the glutaminase gene (kidney type) to be between 8 to 29 fold repeat of GCA. The longest microsatellite correlated with higher glutaminase activity *in vivo.* It increased the risk for overt HE in cirrhotic patients from 20% to 40% (Hazard Ratio 3.12 [CI: 1.39-7.02]; P=0.006)[60]. Furthermore, they carried out a functional analyses that showed how longer forms of the microsatellite promoted higher activity *in vitro*, which implied that it also promoted higher activity of the glutaminase gene, increasing number of glutaminase molecules and thus may enhance glutamine degradation and ammonia production[61]. Therefore, authors concluded that this factor was a genetically

determined difference in the conversion rate of glutamine to ammonia, explaining at least in part the variability in clinical presentation of HE. Mayer et al, confirmed these results in a cohort of 158 patients with liver cirrhosis, most of them in Child B/C. The long-long homozygous form (also called major homozygous) was independently associated with HE irrespective of age or TIPS[62].

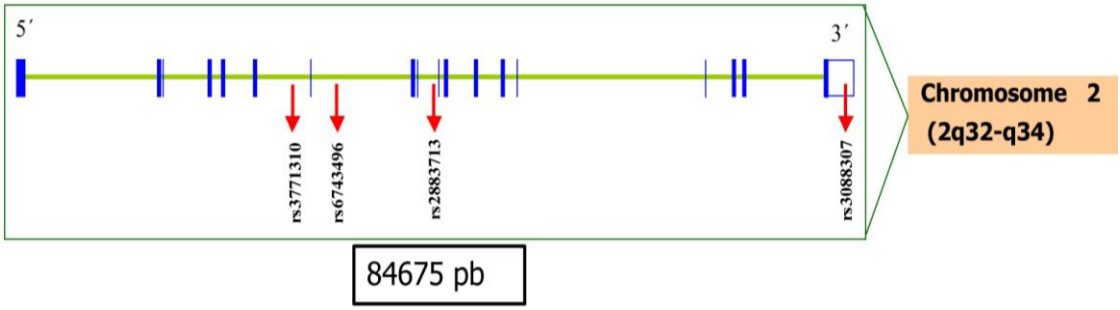

Figure 7. Glutaminase gene is located in chromosome 2.

MAIN DIAGNOSTIC TESTS

In the process of HE, there are two levels of functional impairment which are essential to diagnose, depending on the stage in the HE spectrum: a) impairment in mental status; b) impairment in neuropsychological and neurophysiologic function.

Clinical scales for overt HE

Clinical diagnostic strategies for HE are defective because they are based on subjectivity. West Haven criteria are the most widely used clinical scale. It grades the patient's mental state according to the behavior, intellectual function, alteration of consciousness and neuromuscular function, dividing patients from grade 0, which is normal, to grade 4, which is a coma[63] (**Table 4**). However, results of this scale are not consistent and show poor reproducibility, similar to Glasgow scale (**Table 5**).

	Conscious	Neuropsyquiatric symptoms	Neurological symptoms
Grade 0 (MHE)	Normal	Impairments only detectable by psychometric tests	None
Grade 1	Slight mental impairment	Dysphoria, irritability, anxiety	Fine motor skills disturbed
Grade 2	Fatigue, apathy, letarghy	Subtle personality changes, slight disorientation, inappropriate behavior	Flapping tremor, ataxia
Grade 3	Somnolence to stupor	Agression, gross disorientation	Clonus, asterixis
Grade 4	Coma	None	Signs of increased intracranial pressure

Table 4. Stages of hepatic encephalopathy.

GLASGOW	COMA SCALE	
Eye opening	Spontaneously	4
	To speech	3
	To pain	2
	none	1
Verbal response	orientated	5
	confused	4
	inappropiate	3
	incomprehensible	2
	none	1
Motor response	Obeys commands	6
	Localises to pain	5
	Withdraw from pain	4
	Flexion to pain	3
	Extension to pain	2
	none	1
SCORE (max)		15

Table 5. Glasgow scale.

Neuropsychometric tests for MHE

The psychometric HE score (PHES) is a group of neuropsychometric tests designed to diagnose the typical changes that characterize MHE in cirrhotic patients[64]. Germany, Italy, and Spain are the countries which have validated PHES for the

diagnosis of MHE[65]. It consists of the following tests: Number Connection Test-A (NCT-A), Number Connection Test-B (NCT-B), Line Drawing Test, Serial Dotting Test, and Digit Symbol Test (DST) (**Figure 8**). Depending on the standard derivation range in test results, patients receive points from 0 to -3. The cutoff which determines MHE is −4 points. There are others neuropsychometric tests, like the Repeatable Battery for the Assessment of Neurological Status (RBANS), although are less frequently used[66].

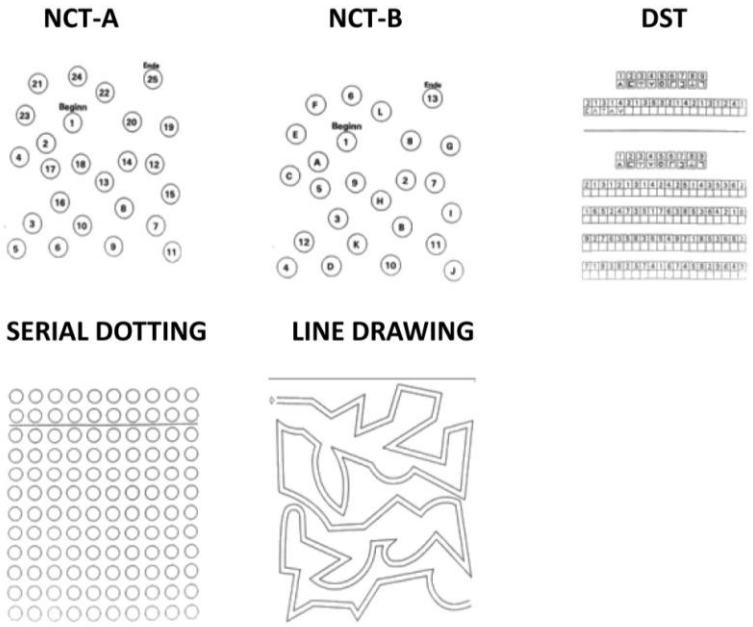

Figure 8. Psychometric hepatic encephalopathy score.

Computerized psychometric tests for MHE

Inhibitory control test (ICT) measures two different cognitive domains, response inhibition and attention, which are affected in patients with MHE[67]. ICT comprises of 1728 stimuli, 40 lures and 212 targets. Worse psychometric performance is associated with a higher lure and lower target rate (a lure response above five indicates MHE with high sensitivity). Similarly, ICT also predicted the development of overt HE, changing

with the clinical status of the patients. Additionally to this, CDR is one of the tests created specifically for patients with MHE[68]. This test measures different mental aspects such as power and continuity of attention or quality of episodic and continuous memory.

Neurophysiologic tests

Critical flicker frequency (CFF) test was validated for the assessment of patients with HE in 2002[69], although was devised originally as an ophthalmological test. The retinal glial cells, in patients with HE, undergo similar changes to those seen in cerebral glial cells, being this fact the basis of CFF. The device causes a stepwise decrease in frequency from 60 to 25 Hz. Main advantages are that the test results are not influenced by sex, occupation or education level, and are only slightly dependent on age. Furthermore, CFF under than 39 Hz diagnoses MHE with high sensitivity and specificity[70].

HE is associated with a decreased mean frequency of electrical activity in the brain, becoming electroencephalography in a good option[71]. The main problem is the needed to be performed under the supervision of a neurologist and require specialized equipment. Additionally, electroencephalography is able to detect the characteristic "triphasic waves" only in advanced HE, although in earlier stages electroencephalography analysis can predict the development of overt HE.

Brain imaging

Cerebral edema in patients with HE is detected by MRI. Many MRI techniques can identify low-grade cerebral edema. On the other hand, there are conditions which could simulate or exacerbate HE symptoms, such as subdural hematoma or a

cerebrovascular event. To prevent these cofounders, a CT scan of the head is a useful test[72].

CLINICAL RELEVANCE

It is well-known that brain dysfunction adversely influences on the quality of life of patients and their performance. However, HE and especially MHE, are often neglected by hepatologists in clinical diary practice. Fortunately, the effect of these syndromes on daily activities is focusing the attention over recent years.

The focus on the relationship between MHE and driving is more than justified, because vehicle accidents are associated with morbidity and mortality, as well as economic and social costs. Cirrhotic patients suffering HE are generally optimistic about their driving abilities. In fact, Kircheis et al. observed that 100% of patients with mild overt HE and 96% of those with MHE were convinced they were good drivers, significantly higher than control subjects (92%), concluding that HE patients overestimated their driving abilities[73]. However, the driving ability of patients with MHE is also reduced as several studies have demonstrated by simulated driving on virtual navigators and on-the-road driving. In fact, Bajaj et al. showed that treating MHE with lactulose is worth in order to prevent driving accidents, being the costs of screening and treating MHE reasonable according to the savings derived from the reduction in accident rates[74].

Cognitive dysfunction, such as HE, predisposes cirrhotic patients to fall as impairs attention and reaction capability[75]. The risk of fracture in cirrhotic patients is higher than general population, thus falls are particularly important in this population. This increased risk has several reasons: a) decreased bone mass due to malnutrition; b) hypogonadism; c) liver insufficiency[76]. Moreover, falls and traumas in cirrhotic patients have considerable complications and mortality. Cirrhotic patients with HE

showed an incidence of falls of 40.4%, in comparison with patients without HE who had an incidence of 6.2%, reaching a 52.3% the probability of falling after 1-year follow-up[77].

TREATMENT STRATEGY

Many treatment options are available for patients with overt HE, while no evidence currently supports the treatment of MHE. In the management of overt HE, it is crucial to identify and correct the precipitating factors. It is common that most of patients show improvement in HE symptoms within 24–48 hours after the initiation of treatment (both empiric therapy and treatment of the underlying causes) (**Figure 9**).

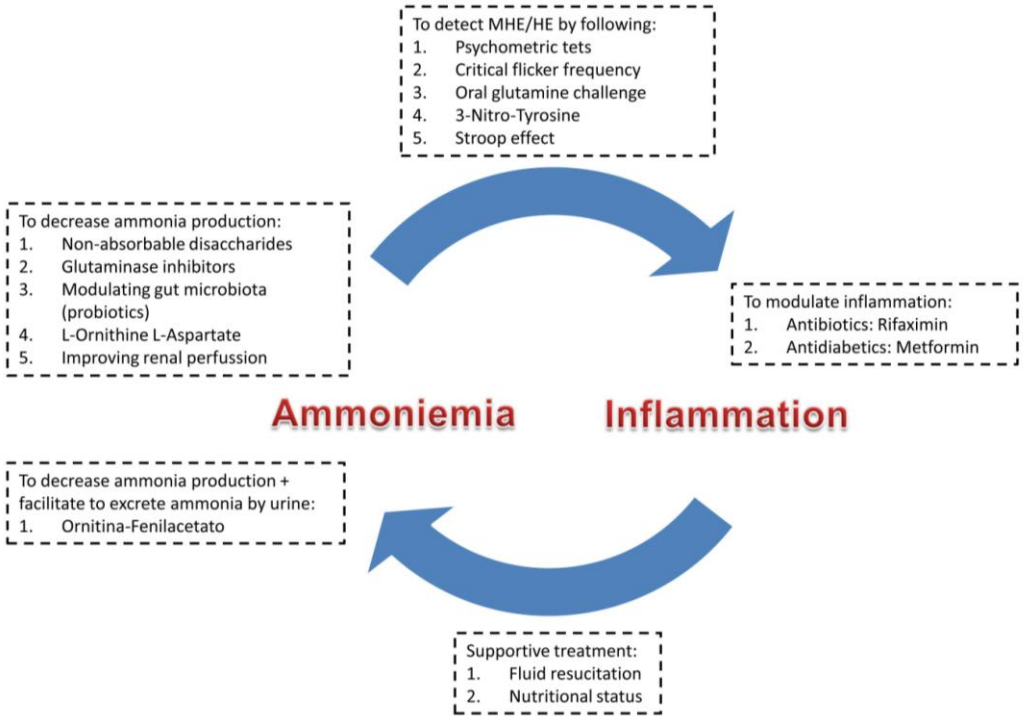

Figure 9. Relevant aspects of the treatment of hepatic encephalopathy.

Non-absorbable disaccharides

Non-absorbable disaccharides (lactulose and lactitol) continue to be the main therapeutic options in the management of HE. In addition to having a laxative effect, lactulose and lactitol promote acidic stools and interfere with mucosal uptake of glutamine in the gut, consequently reducing the synthesis and absorption of ammonia[78].

Antibiotics

Antibiotics have been found to be superior to non-absorbable disaccharides for the improvement of HE[79]. Antibiotics remove ammonia-producing bacterial flora and lower bacterial translocation and systemic inflammation. Rifaximin is a low-gastrointestinal-absorption oral antibiotic with few adverse effects and the systemic drug interaction potential is low. A multicenter study demonstrated that the remission of HE was more prolonged in patients treated with rifaximin compared with those who did not receive it[80]. Neomycin was one of the first antibiotics to be investigated in the field of HE. The use of this antibiotic has declined over the past few years, due to adverse effects such as ototoxicity and nephrotoxicity[81].

Nutritional interventions

Decreasing liver function and increasing porto-systemic shunting increases ammonia concentration, so in this situation skeletal muscle metabolizes ammonia in patients with chronic liver disease. However, cirrhotic patients usually show a loss of muscle mass due to typical the malnutrition status[82]. Consequently, the role of the muscle in detoxification of ammnonia is crucial. In fact, Cordoba et al. found that protein restriction did not improve the clinical evolution of acute HE[83]. Thus, a high-protein diet in cirrhosis status is strongly recommended. The European Society for Parenteral and Enteral Nutrition recommends that cirrhotic patients should eat, at least,

1.2 g/kg of protein daily. They also recommend a diet supplementation with branched-chain amino acids and vegetable protein once HE has developed[84].

Diabetes mellitus

EPIDEMIOLOGY AND CLASSIFICATION

Diabetes mellitus (DM) is a chronic disease that usually appears in two situations: a) the pancreas does not produce enough insulin; b) insulin cannot be used effectively by the body. Accordingly, there are mainly two types of diabetes. On the one hand, type 1 diabetes mellitus (T1DM) is immune-mediated, requiring daily administration of insulin. On the other hand, type 2 diabetes mellitus (T2DM) is characterized by insulin resistance or relative insulin deficiency. T2DM is the most common form, involving 90% of people with diabetes around the world and the prevalence continues increasing (an increase of 20% is expected in developed countries in next ten years). Approximately, 285 million adults, aged 20-79 years, suffer from diabetes mellitus which corresponds to a prevalence of 6.4%[85].

T2DM results from an imbalance between insulin sensitivity and insulin secretion. The alteration of the capacity of the body to respond to insulin effects is the earliest detectable abnormality in the DM spectrum[86]. Consequently, the pancreas increases the insulin secretion to compensate impaired insulin action. During a period of time which varies depending on the individual susceptibility, compensatory hyperinsulinemia maintains glucose level within normal range but β-cells function eventually declines and leads to impaired glucose tolerance and overt diabetes mellitus[87].

INSULIN RESISTANCE

Insulin resistance occurs at an early stage in the development of T2DM (**Figure 10**). There seems to be a continuum from normal glucose tolerance to DM. Insulin resistance leads to increase insulin secretion by the pancreatic β-cell, achieving normal

glucose levels at the expense of elevated serum insulin levels. Ultimately, pancreatic β-cells fail to compensate for the insulin resistance, leading to a condition known as impaired glucose tolerance. Over the years, pancreatic β-cells function deteriorates appearing T2DM. This fact is relevant as insulin resistance not only impairs glucose homeostasis, but is also associated with hypertension, dyslipidemia and abnormalities in coagulation and fibrinolysis. Insulin receptor substrate (IRS)-1 and IRS-2 appear to be the important mediators of insulin signalling in humans. IRS-1 is the first molecule downstream in the insulin-signalling cascade and is specifically involved in skeletal muscle and IRS-2 in adipose tissue insulin signalling. In the liver, impaired insulin signalling from IRS-1 to PKB/Akt leads to increased glucose production *via* inhibition of gluconeogenic enzymes. In addition, glycogen synthesis is inhibited and, at least in rodents, impaired IRS-2 signalling to aPKC leads to increased VLDL synthesis.

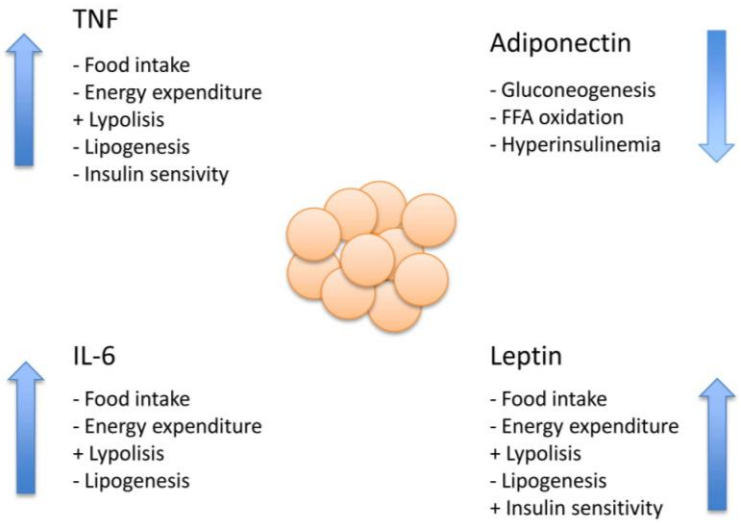

Figure 10. Effects of insulin resistance.

INSULIN SENSITIZERS

Metformin is an insulin-sensitizer drug frequently used in the first-line oral treatment of T2DM patients, belonging to biguanide class. It is generally well tolerated, and its role inducing weight loss has been postulated to be beneficial, as well as the decreasing of low-density-lipoprotein and triglycerides levels. Metformin works increasing beta oxidation of free fatty acids and reducing the hepatic gluconeogenesis via activation of 5'adenosine monophosphate-activated protein kinase (AMPK) pathway; decreasing intestinal glucose absorption; and increasing glucose uptake in skeletal muscle[88]. Furthermore, it is able to modulate the expression of pro-inflammatory cytokines, such as TNFα and IL-6[89]. Recent studies indicate that metformin has antioxidant, anti-inflammatory, growth inhibitory and antiangiogenic effects, reducing the risk of some solid tumors, such as prostate, colorectal, breast and pancreas[90].

Thiazolidinediones (TZD), especially pioglitazone, is another drug class of insulin sensitizers. TZDs act by activating PPARs (peroxisome proliferator-activated receptors), a group of nuclear receptors that regulates gene expression in liver, adipose, vascular endothelium and muscle tissue. They stimulate maturation of visceral fat, and hence change the adipocytokine profile secreted by adipose tissue. TZDs lead to an increase in adiponectin levels, which counteracts pro-inflammatory cytokines such as TNFα and promote beta oxidation of fatty acids via AMPK activation[91].

DIABETES AND LIVER DISEASES

Hepatitis C

Core protein of hepatitis C virus plays a central role in the development of insulin resistance, by mechanisms which depend on viral genotype (particularly, in non-3 genotype)[92]. It inhibits PPAR-γ expression and promotes degradation (by over-expressed TNFα) of IRS-1 and IRS-2, which is balanced with an increase of glycaemia (decreasing glucogenogenesis and increasing gluconeogenesis and liver-related production of glucose)[93]. In fact, when viral clearance is obtained, the expression of IRS-1 and IRS-2 is restored and HOMA-IR index decreases, which indicates the independent role of HCV in insulin resistance[94]. Furthermore, there are other non-structural proteins, like NS5A and NS5B, which also promote insulin resistance, enhancing TNFα and IL-6. Ultimately, the insulin resistance state favors the viral replication, promotes the fibrosis progression and has been associated with lower rates of rapid viral response, as well as sustained viral response[95].

Non-alcoholic fatty liver disease

NAFLD is present when fatty infiltration affects >5% of hepatocytes, in the presence of <20g of alcohol consumption per day, without evidence of other causes of liver disease[96]. Pathophysiology of NAFLD is based in "two-hit hypothesis", which consists in initial accumulation of steatosis in hepatocyte and a second hit, due to oxidative stress, for the development of necroinflammation[97]. Insulin resistance promotes fatty acid accumulation in the liver, resulting in increased β-oxidation and oxidative stress. Increased mitochondrial fat oxidation produces reactive oxygen species and upregulates the nuclear factor kappa-B, which activates the transcription of several pro-inflammatory genes and the production of pro-inflammatory cytokines (TNFα, IL-6 and IL-8)[98]. Furthermore, insulin resistance increases leptin and decreases adiponectin levels. Finally, all these steps lead to NASH.

Fibrosis progression and cirrhosis outcomes

In patients with chronic hepatitis C, studies have shown a significant relationship between the degree of steatosis and severity of fibrosis[99]. It has been suggested that insulin resistance may result from steatosis, as excess free fatty acids could downregulate IRS-1 signalling. Because of steatosis is associated with insulin resistance, fibrosis could be the result of hyperinsulinemia. In fact, it has been demonstrated that high levels of insulin and glucose could promote fibrogenesis by stimulating the release of connective tissue growth factor, a fibrogenic growth factor, from hepatic stellate cells[100].

The risk of spontaneous bacterial peritonitis and ascites is increased in subjects with DM. Sorrentino et al. compared 36 subjects with ascites due to cryptogenic cirrhosis with 108 healthy controls, concluding that ascites was related to different components of metabolic syndrome, such as diabetes mellitus[101]. In this pathology, there is an increased baseline inflammatory activity (via IL-6 and TNFα) and a decreased intestinal motility, which facilitate the bacterial translocation[102]. The role of pro-inflammatory cytokines could be similar in variceal bleeding, as Camma et al. demonstrated in 104 patients with hepatitis C[103]. Regarding to the association between insulin sensitizers and outcomes like spontaneous bacterial peritonitis, ascites and variceal bleeding, there are not studies published yet.

Hepatocellular carcinoma

Diabetes mellitus has been proposed as risk factor to develop HCC in recent studies. Wang et al. have documented in a meta-analysis an increased prevalence of HCC (RR 2.31), as well as mortality (RR 2.43) in subjects with diabetes mellitus[104]. Other studies demonstrated that survival after a HCC curative treatment, recurrence rate[105] and macro-vascular invasion[106] were more often seen in diabetics.

Furthermore, diabetes mellitus has been associated with the presence of metastatic disease among HCC patients[107].

It has been suggested that hyperinsulinemia, caused by insulin resistance, increased levels of insulin like growth factor-1 (IGF-1), which is one of the most potent activators of cellular proliferation via the Akt/mTOR signaling pathway[108]. Insulin also activates the intrinsic tyrosine kinase of insulin receptor, by phosphorylation of IRS-1. Both IGF-1 and IRS-1 are over-expressed in tumor cells, because they generate inhibition of apoptosis[109]. Furthermore, insulin resistance leads to release multiple pro-inflammatory cytokines, including TNFα and IL-6, which promote the development of hepatic steatosis, inflammation and subsequent cancer within the liver[110]. Therefore, since insulin is a growth-promoting hormone with mitogen effects, exogenous insulin and sulphonylureas (which increase serum insulin levels) are considered to enhance carcinogenesis, while metformin that improves insulin sensitivity, decreased the risk of liver cancer development[111]. In fact, metformin use decreases the risk of hepatocellular carcinoma by several mechanisms including: a) phosphorylated AMPK suppresses the Akt/mTOR signaling pathway, inhibiting cell proliferation[112]; b) modulate the expression of cytokines, such as TNFα and oxidative stress[113]; c) antiproliferative and antineoplastic effects associated with inhibition of mTORC1, but the mechanisms are poorly understood.

Antidiabetic therapy is being widely evaluated in HCC. Hassan et al. compared 420 diabetic patients with 1,104 healthy controls (diabetes mellitus was related to HCC (OR 4.2)). They analyzed different treatments, showing metformin and TZD as protective agents (OR 0.3) and sulphonylureas (OR 7.1) and insulin therapy (OR 1.9) as negative factors[114]. Donadon et al. obtained similar results, assessing 610 patients with HCC, 618 cirrhotic patients without HCC and 1,696 healthy controls. Metformin was

shown as protective therapy (OR 0.33), opposite to sulphonylureas and insulin exogenous (OR 3.06)[115]. In other study, 19,349 diabetic patients were compared to 77,396 healthy controls and demonstrated that the prevalence of HCC in diabetic patients was two times higher and that insulin sensitizers were protective (metformin OR 0.49: TZD OR 0.56)[116]. Similarly, Nkontchou et al. observed a reduced incidence of HCC in diabetic cirrhotic patients treated with metformin (HR 0.19)[117].

Aims

Primary aim

-To assess the impact of type 2 diabetes mellitus on the development of hepatic encephalopathy and explore the mechanisms implicated.

-To assess the role of insulin sensitizers on the development of overt hepatic encephalopathy.

Secondary aim

-To assess the role of diabetes mellitus on the diagnostic methods to detect minimal hepatic encephalopathy.

Summary of Results

Several studies had shown a higher prevalence of HE in diabetic cirrhotic patients. Consequently, we aimed to find out the role of insulin sensitizers, particularly metformin, on the prevention of HE. For this purpose, we collected 82 cirrhotic patients with type 2 diabetes mellitus categorized according to metformin treatment; 41 of them were metformin-experienced (average time was 33.4±26.7 months) and 41 were non-metformin-experienced (**Figure 11**). Both cohorts were similar in terms of gender distribution, age and liver function (including Child-Pugh score and MELD score). Etiology of cirrhosis was also similar, being alcohol-related cirrhosis the main etiology. Patients were analyzed according to HE risk score (defined by the presence of altered PHES, CFF and OGC, as well as genetic alterations). During the follow-up, hepatic encephalopathy was diagnosed in 23.2% (19/82) of overall cohort. Main HE triggers were diuretics (31.6%; 6/19) and variceal bleeding (26.2%; 5/19). There was a risk eight-times lower of having HE in patients on metformin treatment compared with non-metformin-experienced patients (4.9% (2/41) vs 41.5% (17/41)) (logRank 9.81; p = 0.002). In multivariate analysis, the metformin use was found independently associated with hepatic encephalopathy [H.R. 0.09 (95% CI: 0.01–0.83); p = 0.034], together with age at diagnosis [H.R. 1.12 (95% CI: 1.04–1.2); p = 0.002], female sex [H.R. 0.10 (95% CI: 0.01-0.67); p = 0.017] and HE risk [H.R. 21.3 (95% CI: 2.8–163.4); p = 0.003]. Additionally, metformin-experienced patients showed a trend to lower TNFr2 levels, compared to non-metformin treated patients, which could indicate the action on pro-inflammatory cytokines. Furthermore, we found that HOMA index was independently associated with higher rate of overt HE in Child-Pugh A patients. This fact is interesting, because supporting the hypothesis that insulin resistance syndrome could promote inflammation and increased risk of overt HE. On the other hand, overall

survival rate reached a trend in metformin-experienced patients: 92.7% (38/41) vs 82.9% (34/41) ($p > 0.05$).

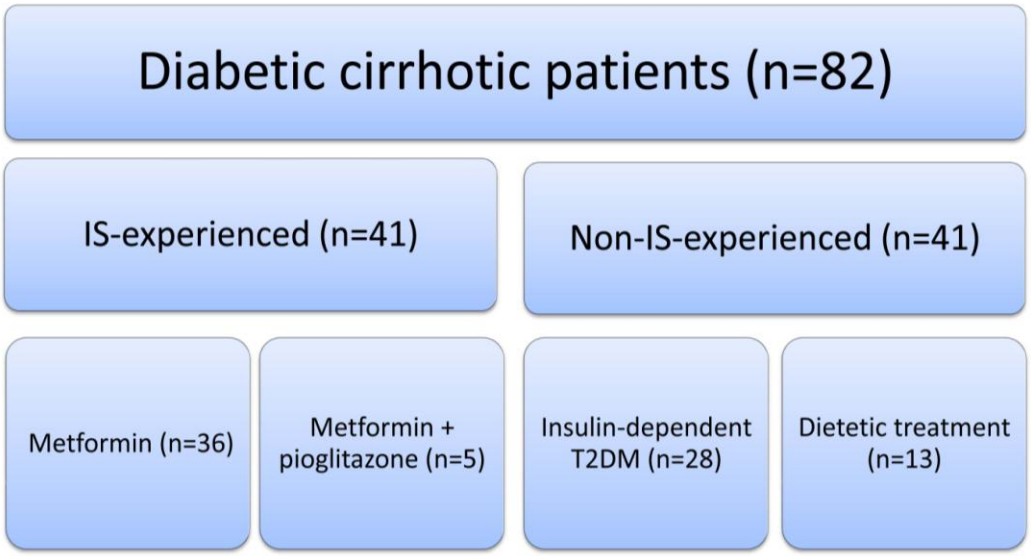

Figure 11. Distribution of patients in case-control study.

On the other hand, we carried out an experimental study *in vitro* to find out the mechanism by metformin could prevent from HE. In the enzymatic assay, different metformin doses (from 0 to 100 mM) were tested with a constant glutamine concentration (100 mM) (**Figure 12**). We observed a dose-dependent reduction of the glutaminase activity with metformin use. Specifically, it was observed 17.5% of glutaminase activity inhibition with a metformin concentration of 10 mM, reaching up to 68% inhibition with higher doses.

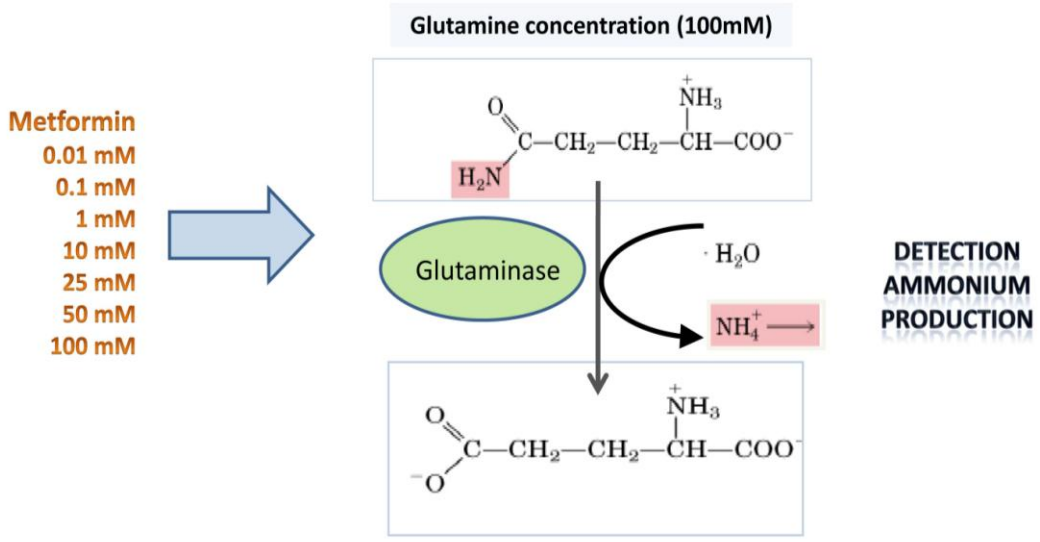

Figure 12. Chemical assay.

On the other hand, Caco2 cells were cultured in presence of different metformin doses (0, 20, 50, 100 and 200 mM) to perform the cells assay (**Figure 13**). Glutaminase activity showed a 24% of inhibition in Caco2 cells with 20 mM of metformin at 72 hours, compared with the control at the same time. Furthermore, we observed a decrease in ammonia production from 26.85±0.74 mM to 19.9±2.05 mM (p=0.05). Other higher metformin concentrations inhibited also the glutaminase activity, but this effect was not different than 20 mM of metformin.

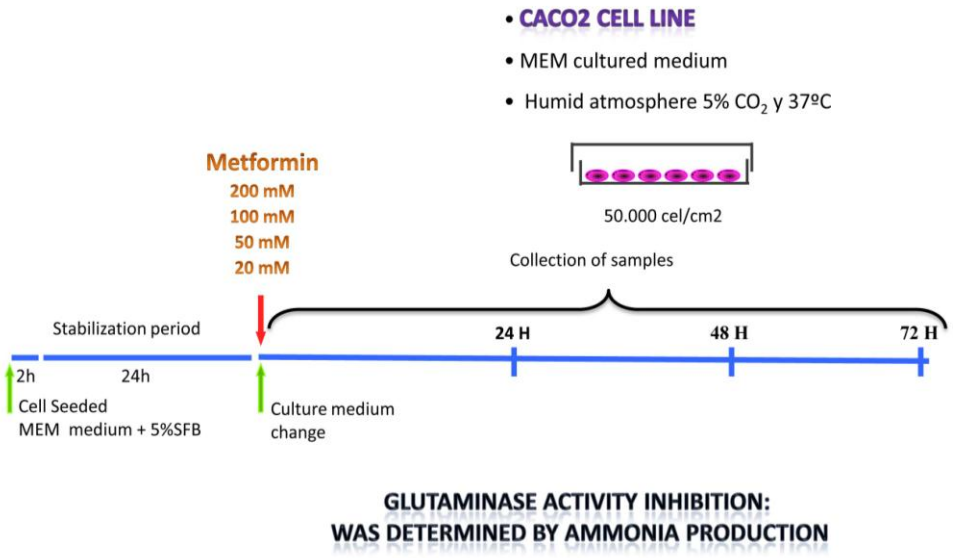

Figure 13. Cells assay.

Additionally to published papers, we have submitted different studies to national and international congresses. One of them consisted in assessing if the presence of diabetes mellitus could alter the results of the different tests to diagnose MHE. Thus, we collected 133 cirrhotic patients, being 36.1% (48/133) diabetics. Liver function did not show any difference between diabetic and non-diabetic patients: a) MELD 9.65 vs 10.35, p=0.357; b) Child-Pugh 6.13 vs 6.35, p=0.400. All cirrhotic patients were undergone to CFF, PHES and OGC as diagnostic tools to detect MHE. All tests detected MHE with similar percentages: 26.3% (35/95) with CFF, 28.6% (38/110) with PHES, and 32.3% (43/133) with OGC. In univariate analysis, diabetes mellitus was not associated with altered PHES (42.1% vs 30.6%; p=0.226) and OGC (37.5% vs 29.4%; p=0.338), but it was related to altered CFF (53.1% vs 28.6%; p=0.019). That is diabetes mellitus increased twice the risk of showing altered CFF. Adjusting by age, sex, MELD and TACC haplotype, the only variable associated independently with altered CFF was diabetes mellitus [O.R. 2.83 (95% CI: 1.17–6.86); p = 0.021].

Discussion

The studies included in this thesis were designed to improve the knowledge and the daily clinical practice in the management of hepatic encephalopathy in cirrhotic patients. Results obtained in our studies allow us to make some general and specific recommendations about the impact of diabetes mellitus on hepatic encephalopathy, as well as the role of metformin on the prevention of this cirrhosis complication.

How could diabetes mellitus promote overt HE?

The prevalence of T2DM and insulin resistance is increasing in Western Countries due to the relationship with obesity and sedentary, so all medical conditions associated with them could be also increasing. Our results showed a closely relationship between T2DM and HE in cirrhotic patients, similar to the previous literature. Butt et al. prospectively enrolled 352 patients with decompensate cirrhosis to assess the impact of T2DM on the prevalence and severity of HE. According to West Haven criteria (it was the diagnostic scale for HE), 50.3% of patients presented HE. They observed that 58.5% of diabetic cirrhotic patients showed HE during the follow-up, while only the 42% of non-diabetic cirrhotic patients developed HE[118]. Similarly, Sigal et al. observed more frequency and more severity of HE in diabetic HCV-cirrhotic patients (in diabetics: 35% mild, 60% severe; in non-diabetic patients: 58% mild, 20% severe) [119]. Taking this special association into account, T2DM and insulin resistance could have a special relevance in the pathogenesis of HE (**Figure 14**). However, there are no studies about mechanisms explaining this association.

Firstly, diabetes mellitus seems to be able to modulate the glutaminase activity. Watford et al. carried out a study with rats which were made diabetic by injection of streptozotocin. They observed that diabetes mellitus caused: a) a slight increase in the glutaminase activity in the kidney; b) a 4-fold increase in hepatic glutaminase activity; c) an increase in the size of the small intestine; d) an increase in the glutaminase activity in the small intestine. When diabetes mellitus was prolonged, the small intestine was visibly enlarged and markedly increased in size (expressed as a percentage of body weight). In these long-term diabetic rats, the glutaminase activity of the small intestine was further increased[120]. As it has been explained above, the increase in the glutaminase activity leads to increase the ammonia production and, consequently, increase the risk of developing HE.

Secondly, diabetes mellitus and insulin resistance are characterized by releasing pro-inflammatory cytokines, such as TNFα and IL-6, which increase the systemic inflammatory response[121]. The synergistic effect of ammonia and inflammation has been proposed, as there is a growing evidence showing the role of inflammation in exacerbating the symptoms of HE. The presence and severity of MHE in cirrhosis is independent of the severity of liver disease and plasma ammonia concentration; however, markers of inflammation are significantly higher in those with MHE compared to those without[122]. In a prospective study in patients showing HE grade 3/4 as the primary indication for ICU admission, those with infection (but not ammonia) develop severe HE. Especially, SIRS score was significantly higher in patients with grade 4 than grade 3. These data highlight the importance of inflammation in severe HE, even without documented infection as 22% of these patients showing SIRS had no positive cultures identified[123]. Furthermore, Odeh et al. studied the relationship between serum levels of TNFα and HE, concluding that there was a positive correlation

with the severity of HE[124]. Similarly, serum levels of IL-6 were found higher in cirrhotic patients with MHE than those without MHE, and also a significant correlation with the severity of MHE was found[125]. During early stages of infection, TNFα is released. It increases the permeability of the blood-brain barrier and favors the diffusion of ammonia into the brain, resulting in HE[126]. Therefore, every pro-inflammatory condition (and diabetes mellitus is one of them) could increase the risk of developing HE.

Thirdly, resistance to insulin actions could promote and increase the protein catabolism and, finally, ammonia production. This effect is consequence of insulin ability to stimulate protein synthesis, as well as inhibit protein degradation in peripheral tissues[127]. On the other hand, patients with liver cirrhosis and diabetes mellitus present malnutrition and often show reduced muscle mass, which could potentially influence on plasma ammonia levels and thereby possibly cognitive function. These findings suggest that low energy intake and poor nutritional status may facilitate the development of HE[128].

Lastly, diabetes mellitus is associated frequently with autonomic neuropathy. As a consequence of the complications in the digestive system, patients with diabetes mellitus may have specific gastrointestinal motility disorders, such as delayed duodenum-cecal transit time, gastroparesis and constipation. These effects can promote SIBO. Diabetes mellitus is implicated in impairments of enteric nervous system, which includes enteric neurons, interstitial cells of Cajal and neurotransmission pathways, as well as on oxidative stress, growth factors and gastrointestinal smooth muscle[129]. Therefore, delayed duodenum-cecal transit in diabetic patients is thought to cause SIBO in this population. In addition, autonomic neuropathy is common in patients with chronic liver disease. Maheswari et al. observed that cirrhotic patients with autonomic

neuropathy were more likely to develop HE due to prolonged intestinal transit time (90% compared to 67%; p = 0.02)[130].

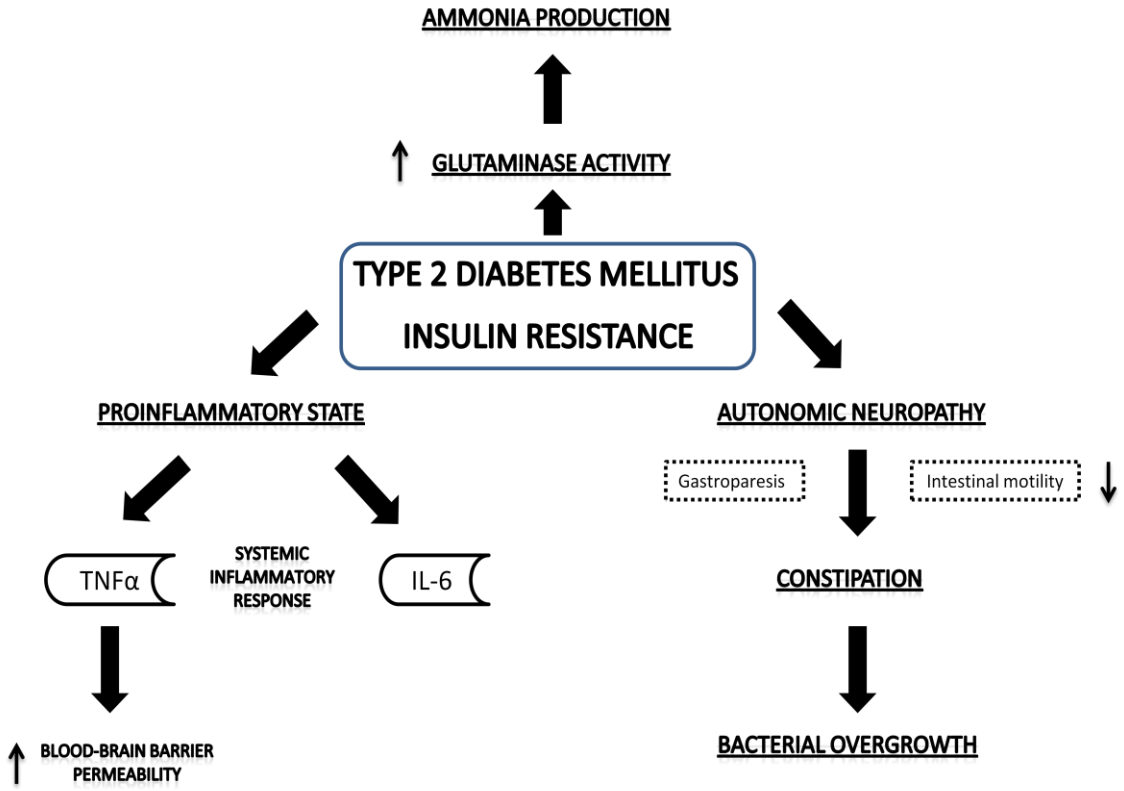

Figure 14. Links between diabetes mellitus and hepatic encephalopathy.

Impact of metformin on the developing of overt HE

Given the fact that diabetes mellitus could increase the prevalence and severity of HE by reasons previously mentioned, we aimed to assess the role of insulin sensitizers (particularly, meftormin) to prevent HE. In our study, we included retrospectively 82 diabetic cirrhotic patients. Cases were defined as patients who were undergone to metformin treatment, while controls were defined as cirrhotic patients with T2DM without metformin treatment. We observed an eight-time lower risk of developing HE in patients on treatment with metformin compared with non-metformin-

experienced patients, despite both cohorts were similar in terms of gender distribution, age, etiology of cirrhosis, liver function (including Child-Pugh score and MELD score) and HE risk score (defined according to altered CFF, PHES, OGC, as well as the presence of genetic alterations).

Metformin is the first choice oral drug for T2DM. It decreases hyperglycemia primarily by suppressing the hepatic gluconeogenesis. In addition to suppressing hepatic glucose production, metformin increases insulin sensitivity, enhances peripheral glucose uptake, decreases insulin-induced suppression of fatty acid oxidation and decreases absorption of glucose from the gastrointestinal tract[131]. We hypothesized three possible mechanisms to explain, at least in part, the protective effect for HE in diabetic cirrhotic patients. First, we obtained a partial inhibition of glutaminase activity, both in the chemical and cells assays when compared with control experiments. To our knowledge, this is the first study to assess the influence of metformin on the glutaminase activity. This enzyme is the main source of ammonia production, so reducing its activity we can prevent from one of the most important triggers (hyperammoniagenic condition). Second, metformin seems to be able to modulate pro-inflammatory cytokines, which promote inflammation by activating macrophages and other immune cells. Metformin has been found to reduce the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) by inhibiting protein and mRNA expression in a dose-dependent manner[132]. Therefore, metformin acts on another clear trigger of HE, the systemic inflammatory response. Third, metformin seems to be able to modify the gut microbiota. In fact, metformin can profoundly affect the fecal microbial community. Metformin increases the number of goblet cells which causes an increase in Akkermansia, bacteria that use the mucus produced by the goblet cells as an energy source. Studies have shown that Akkermansia bacteria improve glucose tolerance and

decreases adipose tissue inflammation. In addition, its administration increases the intestinal levels of endocannabinoids, which has been demonstrated to control the inflammation[133].

The risks and benefits of metformin use in cirrhotic patients with diabetes mellitus are debated. When liver cirrhosis is diagnosed, physicians often discontinue metformin due to concerns about the risk of adverse effects in patients with liver dysfunction[134]. Main concern is metformin-associated metabolic acidosis, which has been largely represented by case reports[135]. However, metformin does not seem to cause or exacerbate liver injury and, indeed, is often beneficial in patients with NAFLD[136], hepatitis C[137] or hepatocellular carcinoma[138]. In a retrospective study, patients who continued treatment with metformin had a significantly longer median survival than those who discontinued the metformin use (11.8 vs. 5.6 years). Furthermore, the continuation of metformin after cirrhosis diagnosis reduced the risk of death by 57%[139]. Our results did not show any serious adverse effect, just some gastrointestinal symptoms such as diarrhea. In prospective studies, the prevalence of gastrointestinal symptoms (e.g. diarrhea) was around 30% of overall cohort[140]. Therefore, metformin could be safety continued in diabetic patients with cirrhosis if there is no specific contraindication.

Impact of diabetes mellitus on the diagnose of MHE

MHE is characterized by a variety of impairments only detected by psychometric or electrophysiological tests in patients with normal mental status[141]. To distinguish MHE from HE grade 1 is a challenge; thus, tests have been proposed to identify the impairment of mental function in patients with earlier stages of the HE

spectrum. These methods explore different pathways and mechanisms of disease, so they are complementary. The prevalence of MHE varies depending on the type of test and the threshold used to define it, being around one third of those with cirrhosis. Our results showed similar MHE detection rates: 26.3% with CFF, 28.6% with PHES, and 32.3% with OGC. We aimed if diabetes mellitus could impact on these diagnostic methods. We found that 53.1% of diabetic cirrhotic patients showed altered CFF, while non-diabetics showed 28.6% (p=0.019).

CFF has been growing in importance in the diagnosis of MHE and to distinguish different stages of the spectrum of HE, as reported in a recent meta-analysis[142]. CFF measures the ability of the central nervous system to detect flickering light. CFF has no contraindications, although we have to be careful in some situations such as patients with migraine (it has been reported to be significantly lower compared with healthy control individuals[143]). Similarly, CFF cannot be implemented in patients with visual defects. According to our results, cirrhotic patients having diabetes mellitus could show frequently an altered CFF. This fact could have several reasons. Firstly, diabetic patients often show visual defects, such as retinopathy[144]. In advanced stages, this entity is obvious and patients are not candidates to CFF. However, diabetic retinopathy could not be easily detected in earlier stages, influencing on the test. P100 wave latency, a type of visual evoked potential, has been found longer in diabetic patients as compared to normal controls, as well as there are a significant reduction in P100 amplitudes in diabetic subjects. Heravian et al. demonstrated that this impairment could be a sign of retinal ganglion cell damage, and could take place before the appearance of the first ophthalmoscopically detectable signs of diabetic retinopathy[145]. Secondly, diabetes mellitus is associated with different brain disorders. CFF assesses the cortical activity[146], and diabetes mellitus has been related to cortical and subcortical infarctions

and, consequently, to mild cognitive impairment[147]. As such, the following mechanisms have been proposed: changes to brain vasculature, disturbances of cerebral insulin signaling, insulin resistance, glucose toxicity, oxidative stress, accumulation of advanced glycation end products and hypoglycemic episodes could be involved in this mild cognitive impairment[148]. In fact, diabetic patients with impaired cognition often show decreased spontaneous brain activity on resting-state functional magnetic resonance imaging[149].

Conclusions

1.- Type 2 diabetes mellitus could increase the incidence and the severity of hepatic encephalopathy.

2.-Type 2 diabetes mellitus could promote hepatic encephalopathy by following mechanisms:

2.1.- Modulating the glutaminase activity.

2.2.- Releasing pro-inflammatory cytokines, such as TNFα and IL-6, which increase the systemic inflammatory response.

2.3.- Promoting and increasing the protein catabolism and, finally, the ammonia production.

2.4.- Promoting delayed duodenum-cecal transit, by autonomic neuropathy, and consequently, causing small intestine bacterial overgrowth.

3.- Metformin seems to prevent from overt hepatic encephalopathy by the following mechanisms:

3.1.- Inhibiting partially the glutaminase activity.

3.2.- Modulating the releasing of pro-inflammatory cytokines.

4.- Metformin is able to modify the effect of diabetes mellitus on the hepatic encephalopathy, decreasing the risk of developing.

5.- Metformin seems to be a safety drug in cirrhotic patients.

6.- Diabetes mellitus could influence on the tests to detect minimal hepatic encephalopathy, especially on critical flicker frequency.

Actividades derivadas de esta tesis

Becas y Premios de investigación conseguidos por el doctorando

-Premio Especial al Mejor Especialista en Formación del Hospital Universitario de Valme de la promoción 2010-2014.

-Beca Dr. Juan Manuel Herrerías en el año 2012 otorgado por la Sociedad Andaluza de Patología Digestiva.

-Beca "Young Investigator" de la International Society for Hepatic Encephalopathies and Nitrogen Metabolism correspondiente al año 2012.

-Beca "Young Investigator (Full Bursary)" de la European Association for the Study of the Liver correspondiente al año 2012.

-Beca "Young Investigator (Full Bursary)" de la European Association for the Study of the Liver correspondiente al año 2013.

-Beca "Young Investigator (Full Bursary)" de European Association for the Study of the Liver correspondiente al año 2014.

-Beca "Joven Investigador" de la Sociedad Española de Patología Digestiva correspondiente al año 2013.

-Beca para la Trainee Course: Summer School 2014 de la United European Gastroenterology, otorgada por la Sociedad Española de Patología Digestiva.

Estancias en Centros Internacionales derivadas de la tesis doctoral

-*Centro*: Hospital of the University of Pennsylvania, Philadelphia, Estados Unidos.

-*Duración*: 3 meses (Septiembre-Noviembre 2013).

-*Responsable*: Rajender K. Reddy.

-*Contacto*: rajender.reddy@uphs.upenn.edu

-*Dirección*: 3400 Spruce St, Philadelphia, PA 19104, Estados Unidos.

Repercusión entre la comunidad científica

-*Ponencia*: “Analytical technologies: new insights”.

-Congreso: 16th Symposium of the International Society for Hepatic Encephalopathies and Nitrogen Metabolism.

-Lugar: Londres, Reino Unido.

-Fecha: Septiembre de 2014.

-*Ensayo clínico*: EME.

- Título: Tratamiento con metformina de la encefalopatía hepática mínima en pacientes con cirrosis hepática.

- Objetivo: Evaluar el efecto de la disminución de la resistencia a la insulina, mediante la administración de metformina, en el tratamiento de encefalopatía hepática mínima, en pacientes con cirrosis hepática.

-Promotor: Fundación Pública Andaluza para la Gestión de la Investigación en Salud de Sevilla.

Anexo I

Relación de las tablas incluidas en la tesis

Anexo II

Relación de las figuras incluidas en la tesis

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